Offsetting Effects of Wolbachia Infection and Heat Shock on Sperm Production in *Drosophila simulans*: Analyses of Fecundity, Fertility and Accessory Gland Proteins

Rhonda R. Snook,*[,1] Sophia Y. Cleland,[†] Mariana F. Wolfner[†] and Timothy L. Karr*

**Department of Organismal Biology and Anatomy, The University of Chicago, Chicago, Illinois 60637 and [†]Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York 14853*

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ABSTRACT

Infection in *Drosophila simulans* with the endocellular symbiont *Wolbachia pipientis* results in egg lethality caused by failure to properly initiate diploid development (cytoplasmic incompatibility, CI). The relationship between Wolbachia infection and reproductive factors influencing male fitness has not been well examined. Here we compare infected and uninfected strains of *D. simulans* for (1) sperm production, (2) male fertility, and (3) the transfer and processing of two accessory gland proteins, Acp26Aa or Acp36De. Infected males produced significantly fewer sperm cysts than uninfected males over the first 10 days of adult life, and infected males, under varied mating conditions, had lower fertility compared to uninfected males. This fertility effect was due to neither differences between infected and uninfected males in the transfer and subsequent processing of accessory gland proteins by females nor to the presence of Wolbachia in mature sperm. We found that heat shock, which is known to decrease CI expression, increases sperm production to a greater extent in infected compared to uninfected males, suggesting a possible link between sperm production and heat shock. Given these results, the roles Wolbachia and heat shock play in mediating male gamete production may be important parameters for understanding the dynamics of infection in natural populations.

THE microorganism *Wolbachia pipientis*, which is closely related to the rickettsiae, is a maternally inherited microorganism found in many arthropod species (Werren *et al.* 1995a,b). Infection alters host reproductive biology in a variety of ways, including feminization (Rousset *et al.* 1992; Bouchon *et al.* 1998), parthenogenesis (Stouthamer *et al.* 1993), and cytoplasmic incompatibility (CI; Yen and Barr 1973; Hoffmann *et al.* 1990; O'Neill and Karr 1990). Unidirectional CI results in reduced egg viability in crosses between infected males and uninfected females (Yen and Barr 1973; Hoffmann *et al.* 1990; O'Neill and Karr 1990; Turelli and Hoffmann 1991; Turelli *et al.* 1992). Crosses between infected individuals, or infected females mated with uninfected males, are reproductively compatible. The inherent asymmetry of CI expression and maternal inheritance of the infection favor the transmission of the bacteria, resulting in an increase in infected females within a population (Turelli and Hoffmann 1991; Turelli *et al.* 1992; Turelli and Orr 1995).

Wolbachia are present in the gametic tissue of males and females (Bressac and Rousset 1993; Karr *et al.* 1998), and antibiotic treatment with tetracycline effectively eliminates the microbe (Glover *et al.* 1990; O'Neill and Karr 1990; Boyle *et al.* 1993). Uninfected individuals arising from antibiotic treatment are reproductively compatible, strongly linking Wolbachia infection and CI expression. Despite this link, very little is known about the cellular and molecular mechanisms by which Wolbachia-induced CI expression is elicited. However, recent studies have found that the expression of CI appears to be a result of Wolbachia disruption of sperm function during and after sperm penetration of the egg. In *Drosophila simulans* (Lassy and Karr 1996) and *Nasonia vitripennis* (Breeuwer and Werren 1990; Reed and Werren 1995), defects in paternal chromosome behavior immediately after sperm penetration have been observed in incompatible crosses. Sperm dysfunction during karyogamy is not due to altered maternal egg factors, as unidirectional CI is only expressed when sperm from an infected male enters an uninfected egg cytoplasm. Furthermore, sperm dysfunction cannot be due to the action of Wolbachia brought into the uninfected egg by infected sperm as Wolbachia are removed from mature sperm during the process of individualization (Bressac and Rousset 1993), and, as shown in this report, Wolbachia are not present in mature sperm within the seminal vesicle. The compatibility asymmetry and the removal of Wolbachia from sperm

Corresponding author: Dr. Timothy L. Karr, Department of Organismal Biology and Anatomy, 1027 E. 57th St., The University of Chicago, Chicago, IL 60637. E-mail: tkarr@midway.uchicago.edu

[1]*Present address:* Department of Biological Sciences, University of Nevada, Las Vegas, NV 89154-4004.

before fertilization suggests the mechanism affecting CI expression is twofold: first, Wolbachia acts before the completion of spermatogenesis and, second, infected eggs "rescue" the Wolbachia-induced defect (sperm from infected males are only compatible with infected eggs).

Wolbachia imposes a physiological cost to females in that infected females oviposit fewer eggs than uninfected females (Hoffmann *et al.* 1990, 1998; Nigro 1991). The mechanism by which Wolbachia elicits this cost is unknown. However, on a relative basis, this cost is not realized because, although uninfected females lay more eggs, they produce fewer offspring due to CI expression when mated to infected males.

Wolbachia's influence on male fitness, through actions on sperm production and associated seminal fluid components, has not been studied. Male reproductive success in Drosophila can be affected by a number of factors, including the number of sperm and the amount and composition of seminal fluids transferred to females. A positive relationship between the female remating interval and both the number of sperm used (measured by the number of progeny a female produces) and the number of sperm received per copulation has been described for some Drosophila species (Gromko *et al.* 1984; Schwartz and Boake 1992; Pitnick and Markow 1994), including *D. simulans* (Gromko and Markow 1993). The female remating interval influences the level and outcome of sperm competition and thus a male's fitness (for review see Simmons and Siva-Jothy 1998). Seminal fluid molecules called accessory gland proteins (Acps) have also been shown to affect male fitness by their influence on postcopulatory interactions with females. For example, Acp26Aa induces ovulation in newly mated females (Herndon and Wolfner 1995; Heifetz *et al.* 2000), Acp70A (sex peptide) also can increase egg laying (Chen *et al.* 1988; Aigaki *et al.* 1991), and the transfer of Acp36DE is required for proper sperm storage by females (Neubaum and Wolfner 1999; Tram and Wolfner 2000). Some Acps reduce female receptivity to courting males (Chen *et al.* 1988; Aigaki *et al.* 1991), which subsequently decreases the level of sperm competition. Variance in four Acp loci has been observed that correlated with the efficiency of sperm competition (Clark *et al.* 1995).

Male fertility and CI may also be influenced by heat shock and the subsequent endogenous production of heat-shock proteins (Hsps). For example, Hsp26, 70, and 90 are expressed during spermatogenesis in Drosophila (Glaser *et al.* 1986; Yue *et al.* 1999) and Hsp70 and 90 are present in mature sperm (Feder *et al.* 1999; Yue *et al.* 1999). Reduced Hsp90 expression results in the production of sterile males, as a likely consequence of the involvement of Hsp90 in altering signal transduction pathways that control microtubule function (Yue *et al.* 1999). Heat shock is also an ecologically relevant parameter occurring in natural populations (Feder *et al.* 1999). Brief heat shock of laboratory populations of *D. simulans* reduces CI expression (Feder *et al.* 1999), suggesting that transient increases in Hsp expression may, either directly or indirectly, ameliorate Wolbachia's effect on sperm. However, other mechanisms associated with brief heat shock may also be consistent with CI suppression. For example, the severity of CI expression is known to be related to Wolbachia levels (Boyle *et al.* 1993; Breeuwer and Werren 1993), and the heat shock may simply reduce Wolbachia numbers, consequently reducing CI expression.

Here we examine and compare sperm production and accessory gland protein expression and processing between laboratory strains of infected and uninfected *D. simulans*. We also examine how heat shock impacts sperm production in infected and uninfected males. We report that, similar to infected females, Wolbachia infection imparts a physiological cost of infection on males by significantly reducing sperm numbers. Consistent with these findings, infected males have reduced fertility relative to uninfected males in different mating scenarios. Conversely, we demonstrate that heat shock may abate the physiological cost of infection and its subsequent male fertility effects by dramatically increasing sperm production. Thus, the previously demonstrated reduction in CI expression by heat shock (Feder *et al.* 1999) may be related to the increase in sperm production elicited by heat treatment.

We also eliminated two other possible effects of Wolbachia on fertility and reproductive biology: (1) Wolbachia do not affect either the transfer by males or processing in females of Acp26Aa and Acp36DE, two accessory gland proteins known to affect female fertility; and (2) Wolbachia are absent from mature sperm in seminal vesicles and therefore cannot affect sperm function once transferred to females. Thus, the fertility effect on males is due to the direct effect of Wolbachia on sperm production rather than any effect of Wolbachia on Acps or mature sperm. These results highlight the continued need for detailed laboratory and field studies to understand the distribution of Wolbachia, the costs and benefits to Wolbachia and its host, and the mechanisms contributing to variable CI expression patterns in nature.

MATERIALS AND METHODS

Fly stocks and culture: All stocks were maintained on cornmeal-molasses-agar food or, for Acp analyses, on yeast/glucose food and a 12:12-hr light/dark cycle at 25°. An infected strain of *D. simulans* (DSR) was originally collected from Riverside, California (kindly provided by M. Turelli). The uninfected stock (DSRT) was derived from DSR after tetracycline treatment as described (O'Neill and Karr 1990). Absence of infection after antibiotic treatment was determined as described previously (O'Neill and Karr 1990; O'Neill *et al.* 1992). For each experiment, larval density and access to yeast were controlled by collecting first-instar larvae from eggs ovi-

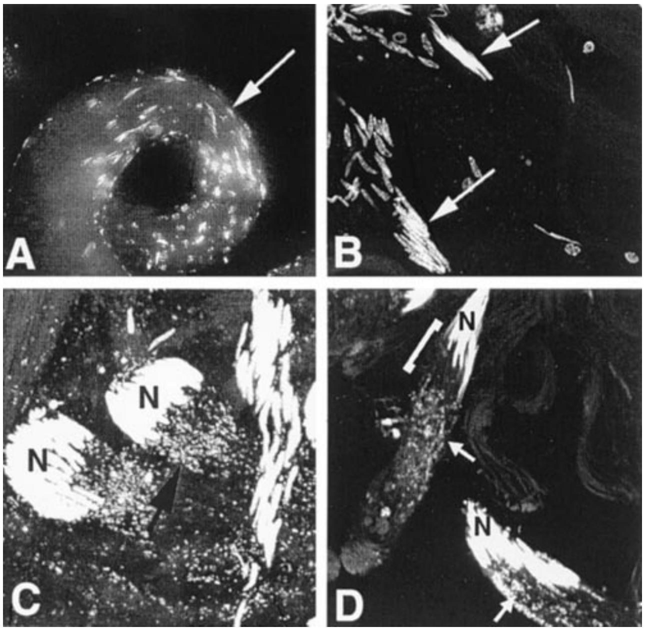

Figure 1.—V cysts and Wolbachia distribution during spermatogenesis in *D. simulans.* (A) Low-magnification view of V cysts in distal region of testis (arrow points to one V cyst). (B) Confocal higher magnification view of V cysts (arrows point to the nuclei in two cysts). (C) Wolbachia distribution in young spermatocyst clustered proximal to the cyst nuclei. (D) Wolbachia distribution in successively older sperm cysts (arrows). Wolbachia in upper, older cyst, are displaced away from nuclei (N).

posited onto molasses-agar plates. Fifty first-instar larvae were placed in vials containing 10 ml of standard cornmeal-agar food. Adults from these vials were collected within 4 hr of eclosion and 10 same-sex individuals were placed in food vials supplemented with 0.001 g dry yeast.

Effect of infection on sperm production: We measured sperm production with respect to infection status. Because *D. simulans* sperm are *ca.* 1.20 mm in length (Hihara and Kurokawa 1987; Karr 1996), accurate counts of mature sperm are extremely difficult and time consuming. As an index of the number of sperm produced, we quantified the number of sperm bundles at a characteristic stage of spermatogenesis that we denote the "V-cyst" stage and describe below.

Spermatogenesis occurs in an elongated cylindrical testis and proceeds from the apical tip toward the distal end, where mature sperm are delivered to the seminal vesicle (Figure 1A). At the apical end of the testes, stem cells divide to produce a spermatogonial cell that undergoes four rounds of mitosis to produce 16 primary spermatocytes. Subsequent meiosis results in developing cysts containing 64 immature sperm. During the final stages of sperm cyst elongation, sperm nuclei elongate, forming needle-like structures. As nuclear condensation occurs, these nuclei align at the apical end of the cyst. These shape changes cause cyst nuclei to form a characteristic V-shape that is easily recognized in 4′,6-diamidino-2-phenylindole (DAPI)-stained preparations (examples of the range of structures used as search images are shown in Figure 1, B and C). Spermatogenesis proceeds linearly (Fuller 1993), and therefore each developing cyst must pass through the distinct V-cyst stages. Thus the number of V cysts represent a convenient, easily identifiable, and accurate index of sperm numbers.

To determine V-cyst numbers, virgin infected (DSR) and uninfected (DSRT) males were collected and stored as described above. After either 1, 3, 5, 7, or 10 days after eclosion, the males testes were dissected in phosphate-buffered saline (PBS; 0.05 m sodium phosphate, 0.1 m sodium chloride, pH 7.8), transferred to PBST (PBS containing 0.1% Triton X-100), and either (1) fixed in 3.7% formaldehyde in PBST for 20 min or (2) placed under a coverslip and rapidly frozen in liquid nitrogen and subsequently fixed in acetic acid/methanol. Fixed testes were rinsed in PBS and stained with DAPI (Sigma, St. Louis). Fixed and stained testes were mounted on microscope slides under coverslips in a mounting medium containing glycerol:PBS (80:20, v/v), respectively, containing 1% *n*-propylgallate (w/v; Giloh and Sedat 1982). Testes were examined using an Axioplan2 microscope (Zeiss, Thornwood, NY) equipped with epifluorescence and Nomarski DIC optics. Images were visualized using the appropriate filters for DAPI fluorescence and a ×40 Plan Neofluar lens (n.a. 0.75). Sperm cysts were counted manually by examining the distal end of the testes near the seminal vesicle entrance (Figure 1A). Representative images of V cysts used in the analysis of sperm production (Figure 1, A, B, and D) were generated by digital deconvolution (Vaytek, Inc., Fairfield, IA) of optical sections along the *z*-axis using a 12-bit CCD camera (Princeton Instruments). The sample sizes of infected and uninfected males ranged from 21 to 25 for each day after eclosion they were examined.

Effect of heat shock on sperm production: We determined how heat shock influenced sperm production with respect to infection status using the following protocol. Fifty first-instar larvae from either DSR or DSRT were placed in a vial containing 10 ml of food. Male third-instar larvae were subsequently collected from these vials and placed in a sealed glass vial containing a moist paper towel to preserve humidity. The vial was placed in a 36° water bath for 150 min. After heat treatment, larvae were placed in food vials (50 larvae per vial) containing 10 ml standard food. Heat-shocked (hsDSR) and (hsDSRT) males were collected and stored as described above. Testes dissections, processing, and counting occurred as described above. The number of males investigated ranged from 16 to 25 for each day after eclosion they were examined.

We used a two-way analysis of variance with treatment (DSR, DSRT, hsDSR, and hsDSRT) and days (1, 3, 5, 7, and 10) as main effects to determine whether there were any differences between males in the number of sperm cysts produced. Tukey post-hoc comparisons were performed when appropriate.

Effect of male mating on male fitness: We determined the effect of male mating on male fitness in two different experiments. First, we examined the effect of constant interaction between single pairs of flies on male fitness. Virgin females and males were collected from density-controlled vials and stored as described above. Three-day-old males were individually placed with an 8-day-old female for 5 days. The number of times a single pair mated for the 5 days is unknown. The following four crosses were performed: DSR × DSR (female × male; compatible), DSRT × DSRT (compatible), DSR × DSRT (compatible), and DSRT × DSR (incompatible). Pairs were maintained together in an egg-laying manifold with 20 individual chambers, each chamber containing food and substrate for egg laying for a single pair (Karr *et al.* 1998). Food plates on the manifolds were replaced every 24 hr throughout the 5 days of the experiment. The eggs in each chamber were counted immediately after the plates were removed. The unhatched eggs were counted 24 and 36 hr after plate removal. Egg counts from the first 24 hr after mating were discarded due to the low number of eggs oviposited. Data for the four subsequent days were kept; these are referred to as days 1–4. Females laying less than a total of 10 eggs on any day were excluded from the analysis. We used the proportion of unhatched eggs from a male's partner as an index of male fitness. A one-way analysis of variance was performed to determine male fitness effects under condition of single pair mating. Tukey post-hoc comparisons were performed when appropriate. Sample sizes are presented in Figure 3.

Second, we examined the role of sequential male mating in male fitness. Virgin females and males were reared and stored as described above. Two crosses, DSR × DSRT and DSR × DSR, were performed. Only DSR females were used to control for the effect of Wolbachia infection on egg production (DSR females produce fewer eggs than DSRT females) and incompatibility (DSRT females mated with DSR males are subject to CI). Three-day-old males were sequentially mated to three 7-day-old virgin females within a 6-hr period. Only males that had been observed to mate three times within the 6-hr period were used in the subsequent male fitness assay. At 6 hr postsequential mating, males were placed singly in a food vial with a virgin female. Copulation was observed, after which the mated females were transferred to egg-laying manifolds. To stimulate egg laying in the absence of males, manifolds were preconditioned with males for 24 hr (Hoffmann 1985; Habrova *et al.* 1996) before mated females were placed in the manifold. The number of eggs oviposited and the number of unhatched eggs were counted as described above, with the first day being discarded and females laying less than a total of 10 eggs on any day excluded from the analysis. Differences between treatments in the total proportion of unhatched eggs were tested using a *t*-test. We performed a repeated measures analysis of variance with appropriate post-hoc tests to examine the temporal pattern of fitness costs of multiply mated males. Sample sizes are presented in Figure 4.

Statistical analyses were performed using either Systat 5.0 (SPSS, Inc.) or JMP 3.0 (SAS, Inc).

Polymerase chain reaction (PCR) and fluorescent analyses of mature sperm: Testes with attached seminal vesicles (from 3- to 5-day-old virgin males) were dissected, and the seminal vesicle was removed carefully into a drop of sterile PBS on a microscope slide and then rinsed three times in fresh, sterile PBS. The vesicle was then placed in a fresh drop of sterile PBS, pierced with a sterile dissecting needle, and sperm were removed by "spooling" onto sterile forceps and subsequently transferred to a 1.5-ml microcentrifuge tube containing PBS. Sperm from either five DSR or DSRT males were collected in this manner and transferred into 20 μl of 50 mm Tris-HCl, pH 8.0; 20 mm NaCl; 1 mm EDTA; and 1% SDS. One microliter of Proteinase K (11 mg/ml; Sigma) was added, and the mixture was incubated for 30 min at 55°, vortexed vigorously for 15 sec, and incubated for 30 min. After incubation, 120 μl H_2O was added, mixed by vortexing briefly, and heated to 100° for 10 min. One microliter of this solution was used in each PCR amplification. Testes from the same DSR males whose sperm was used were treated in a like manner, and 1 μl of this solution served as a positive control. PCR was carried out using Wolbachia-specific (16S rRNA) and mitochondrial-specific (12S rRNA) primers essentially as described (O'Neill *et al.* 1992). Each reaction had a final volume of 50 μl containing 50 mm KCl; 10 mm Tris-HCl, pH 9.0; 0.1% Triton X-100; 2 mm $MgCl_2$; 5% DMSO; 0.2 mm dATP, dCTP, dGTP, and dTTP; and 1.5 units of *Taq* polymerase (Promega, Madison, WI). Conditions for PCR conditions were as follows: 2 min at 92°, followed by 45 cycles of 92° for 30 sec, 50° for 30 sec, 72° for 50 sec, followed by 2 min at 72°.

Sperm were analyzed for the presence of Wolbachia by confocal microscopy using the DNA-specific dye Sytox Green (Molecular Probes, Eugene, OR) essentially as described (Kose and Karr 1995). Sperm from DSR and DSRT males were dissected from seminal vesicles as described above, transferred to a glass microscope slide, air-dried, and subsequently stained with a 0.1-μg/ml solution of Sytox Green in PBS buffer.

TABLE 1

Analysis of variance for effect of treatment (DSR, DSRT, hsDSR, hsDSRT) and day (1, 3, 5, 7, 10) and the interaction between these main effects on the number of sperm cysts produced

Source	d.f.	MS	*F*	*P*
Treatment	3	2901.8	46.1	<0.001
Day	4	4675.8	74.3	<0.001
Treatment × day	12	307.5	4.89	<0.001
Error	410	62.9		

Effect of infection status on Acp26Aa and Acp36DE: Virgin male and female flies were collected from the DSR and DSRT stocks and aged separately until they were 3–5 days old. Females were then mated either to males of their own strain or to males from the other strain. Proteins from 10 dissected male accessory glands, from 10 dissected genital tracts of unmated females, or from 10 mated females collected ~30 min after the start of mating were extracted, electrophoresed on 10% SDS-PAGE, and processed for Western blotting as described in Park and Wolfner (1995). Blots were probed sequentially with affinity-purified anti-Acp26Aa (Monsma and Wolfner 1988) and anti-Acp36DE (Bertram *et al.* 1996) directed against *D. melanogaster* Acp 26Aa and 36DE.

RESULTS

Effect of Wolbachia infection and heat shock on sperm production: Sperm production was estimated by counting maturing spermatids at the V-cyst stage of development (see Figure 1, A and B, for search images). Two-way analysis of variance revealed robust effects of both treatment (DSR, DSRT, hsDSR, and hsDSRT) and days (1, 3, 5, 7, and 10) on sperm production (Table 1; Figure 2). Tukey post-hoc pairwise comparisons for the treatment effect revealed that across all days, DSR males produced the fewest number of V cysts, followed by hsDSR males, with both DSRT and hsDSRT males containing equivalent V cysts over the course of the experiment (Figure 2). Tukey post-hoc pairwise comparisons for the day effect revealed that 1-day-old males produced fewer V cysts than at any other age (Figure 2). Three-day-old males contained more V cysts than 1-day-old males but fewer than when they were 5 and 7 days old (Figure 2). Five- and 7-day-old males produced equivalent V-cyst numbers. Interestingly, V-cyst production in 10-day-old males decreased back to levels equal to those of 3-day-old males (Figure 2).

In addition to highly significant main effects, a significant interaction between treatments and days was found (Table 1; Figure 2). This interaction is a result of several different effects on males. Throughout the experiment, DSR males tended to produce the fewest sperm cysts. Most strikingly, the rate of change in V-cyst production was generally slower in infected males. For example, DSRT males increased the number of V cysts by 42% between days 1 and 3, whereas DSR males only

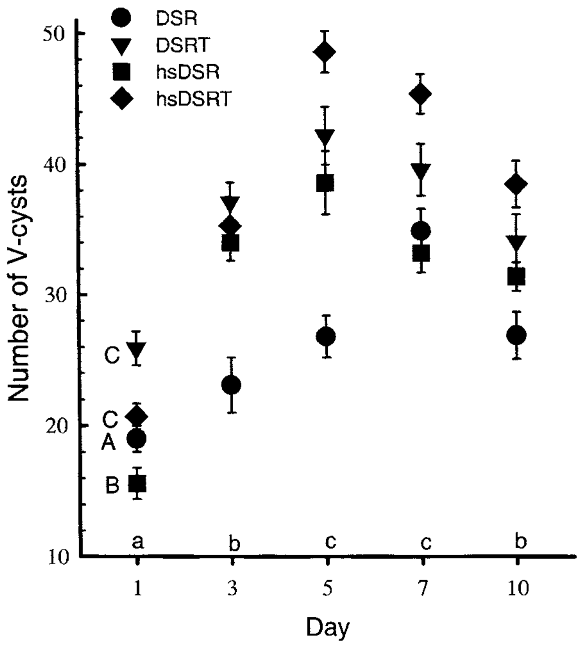

Figure 2.—The number of V-cyst sperm produced by virgin DSR, DSRT, heat-shocked DSR (hsDSR), and heat-shocked DSRT (hsDSRT) males for 5 different days following eclosion. Lowercase letters on the abscissa indicate days that are significantly different from each other as determined by two-way analysis of variance and Tukey's post-hoc pairwise comparisons. Capital letters on the ordinate indicate treatments that are significantly different from each other as determined by two-way analysis of variance and Tukey's post-hoc pairwise comparisons.

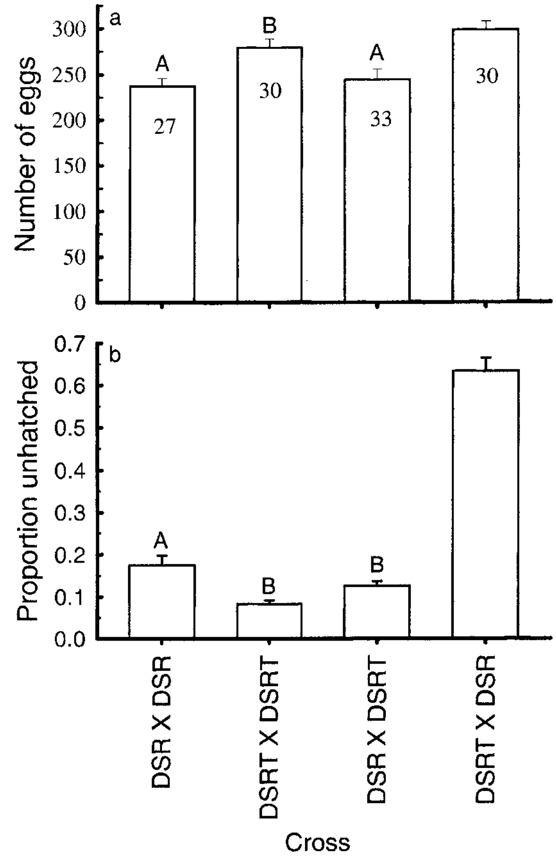

Figure 3.—The number of eggs (a) and proportion of unhatched eggs (b) for each treatment in which virgin females were mated with virgin males. Numbers in bars are sample sizes for each treatment. Capital letters indicate treatments that are significantly different from each other as determined from analysis of variance and Tukey's post-hoc pairwise comparisons.

increased production by 22%. Relatively then, 3-day-old DSR males produce only 62% as many sperm cysts as DSRT males. This difference is further increased on day 5, when DSR males produce only 57% as many sperm cysts as DSRT males. Sperm cyst production in DSR males does not equal that of DSRT males until day 7. Additionally, on day 10, while both DSR and DSRT males produce fewer sperm cysts than on day 7, the effect on DSR males is more pronounced. DSR males reduce sperm cysts by 30% compared to 16% in DSRT males. Similar differential effects of heat shock on sperm production were seen. One-day-old hsDSR males produced significantly fewer sperm cysts than any other type of male, including DSR. However, by day 3, hsDSR males have dramatically increased sperm production above DSR and are producing numbers of sperm cysts equivalent to those of hsDSRT and DSRT. Heat-shock treatment also increased sperm production in uninfected males. On days 5 and 7, hsDSRT males produced significantly more sperm cysts than hsDSR, DSRT, or DSR males. By day 10, all males produced equivalent sperm numbers. While heat-shock treatments elevated the number of sperm cysts for both hsDSR and hsDSRT relative to their non-heat-shocked counterparts, heat shock tended to have more of an effect on DSR males. Relative to their non-heat-shocked counterparts, infected males increased sperm production by 17% compared to 5% for uninfected males.

Wolbachia in sperm cysts were also examined during different stages of maturation (Figure 1, C and D). Wolbachia clustered near nuclei (N, Figure 1C) in immature spermatocysts (arrow, Figure 1C). The developmental age of individual spermatocysts can be estimated by the degree of sperm nuclear compaction and elongation (N, Figure 1, C and D). At later stages of maturation during sperm individualization (Figure 1D) Wolbachia were observed to migrate away from nuclei in older (arrowhead, Figure 1D) compared to younger (arrow, Figure 1D) sperm. Wolbachia are presumably excluded from individual sperm by the advancing sperm membranes that form during the individualization process.

Effect of male mating on male fitness: In the first mating experiment, single pairs were placed together for 5 days. Ignoring the incompatible cross, we found that infected females oviposited fewer eggs than uninfected females (Figure 3a; $F = 4.858$; d.f. $= 2, 87$; $P < 0.01$), as reported in other studies (Hoffmann *et al.* 1990; Nigro 1991). The incompatible cross resulted in

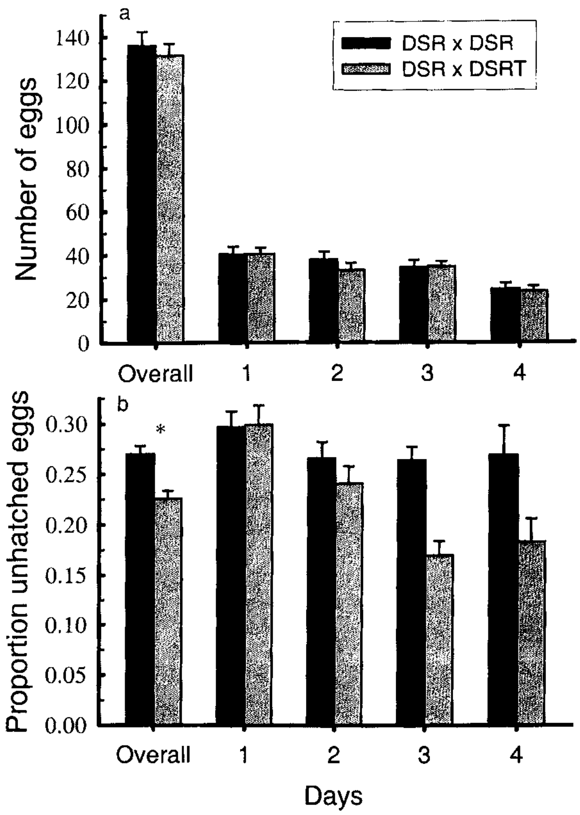

Figure 4.—The number of eggs (a) and proportion of unhatched eggs (b) from virgin DSR females mated to previously sequentially mated DSR (DSR × DSR) or DSRT (DSR × DSRT) males. (*) Significant differences between treatments (DSR × DSR *vs.* DSR × DSRT) as determined by a *t*-test (overall).

~75% egg lethality (Figure 3b). DSRT males produced more progeny than DSR males when both were mated to compatible (infected) females whereas DSRT males produced similar progeny numbers regardless of the infection status of the female (Figure 3b; $F = 9.8205$; d.f. = 2, 87; $P < 0.0001$).

In the second experiment, we examined the effect of sequential male mating on male fitness. We found that DSR females mated to sequentially mated males of different infection status oviposited the same total number of eggs (Figure 4a; $t = 0.646$, d.f. = 46, $P = 0.521$). However, females mated to sequentially mated DSR males produced a greater total proportion of unhatched eggs than females mated to DSRT males (Figure 4b; $t = 4.366$, d.f. = 46, $P < 0.001$). The proportion of unhatched eggs in matings with infected males was 19.8% greater than matings with uninfected males (Figure 4b). The largest difference in fertility between infected and uninfected males occurred on the third day after mating. For the third and fourth days, the combined fitness of infected males was decreased by 33% compared to uninfected males.

Repeated measures analysis of variance further confirmed that females mated to males of different infection status did not differ in egg production (Table 2a, Between-subjects effects). Within-subject effects were found to be significant, indicating that females oviposited decreasing numbers of eggs across time, but this effect was not dependent on the infection status of males (Table 2a; Figure 4a). Repeated measures analysis of variance also confirmed that females mated to males of a different infection status significantly differed in the proportion of unhatched eggs (Table 2b, Between-subjects effects). Within-subject effects were also significant, indicating that females had a smaller proportion of unhatched eggs across time that was dependent on the infection status of the male mate (Table 2b; Figure 4b). Thus, DSR females mated to DSR males had a larger proportion of unhatched eggs (implying lower fitness for infected males) and this proportion remained relatively high compared to DSR females mated to DSRT males (Table 2b; Figure 4b). Single degree of freedom polynomial contrasts suggested this decrease in the proportion of unhatched eggs occurred linearly, with the DSR × DSRT cross having a greater response (Table 2b; Figure 4b). Subsequent univariate *F*-tests indicated that the linear decline in the proportion of unhatched eggs (and thus linear increase in male fertility) in the DSR × DSRT cross occurred between days 1 and 2 and days 2 and 3, but stayed similarly low between days 3 and 4 (Table 2b; Figure 4b).

Wolbachia are absent from mature sperm: Large numbers of Wolbachia are present in developing spermatids (Figure 1) and appear to be excluded from sperm during the individualization process (Bressac and Rousset 1993). To determine if Wolbachia are absent from mature sperm in the seminal vesicle, Wolbachia-specific primers and PCR (O'Neill *et al.* 1992) were used to assay mature sperm for the presence of Wolbachia (Figure 5A). As expected, a single band of ~1 kb was amplified from infected male testes (Figure 5A, lane 1); this band was absent from DSRT testes (Figure 5A, lane 2). No positive PCR signal was detected from mature sperm by this assay (Figure 5A, lane 3). To ensure that the PCR assay worked in these samples, 12S rDNA-specific primers were used to amplify a portion of the mitochondrial 12S rDNA gene (O'Neill *et al.* 1992). As expected, a single band of ~700 bp was detected in all samples (Figure 5A, lanes 1, 3, and 4).

We also searched for Wolbachia in mature sperm using fluorescence microscopy and a DNA-specific dye to search for the characteristic punctate staining characteristic of Wolbachia (Figure 1; Glover *et al.* 1990; Boyle *et al.* 1993; Bressac and Rousset 1993; Kose and Karr 1995). No DNA-positive structures characteristic of Wolbachia were observed in over 2000 sperm examined from either 10 DSRT or 10 DSR males (Figure 5B).

Acp26Aa is produced, transferred, and processed normally in DSR and DSRT: In *D. melanogaster*, Acp26Aa appears on Western blots as a triplet of bands (37–41

TABLE 2

Repeated measures of analysis of variance of DSR females mated to either previously mated DSR or DSRT (treatment) for the number of eggs (a) and proportion of unhatched eggs (b) produced with appropriate post-hoc tests

Source	MS	*F*	*P*
(a) No. of eggs produced			
Between-subject effects (d.f. = 1, 46)			
Treatment	88.021	0.418	0.521
Error	210.634		
Within-subject effects (d.f. = 3, 3, 138)			
Eggs	2232.5	10.202	<0.001
Egg × treatment	62.243	0.284	0.837
Error	218.82		
(b) Proportion of unhatched eggs			
Between-subjects effects			
Treatment (d.f. = 1, 46)	0.124	17.791	<0.001
Error	0.007		
Within-subjects effects (d.f. = 3, 3, 138)			
Unhatched eggs	0.063	7.093	<0.001
Unhatched eggs × treatment	0.026	2.958	0.035
Error	0.009		
Single degree of freedom polynomial contrasts: Order 1 (linear)			
Unhatched eggs	0.152	14.125	0.001
Unhatched eggs × treatment	0.067	6.20	0.016
Error	0.011		
Univariate *F*-tests: Variable			
Day 2–1 (d.f. = 2, 46)	0.052	3.483	0.039
Error	0.015		
Day 3–2 (d.f. = 2, 46)	0.061	5.338	0.008
Error	0.011		
Day 4–3 (d.f. = 2, 46)	0.002	0.118	0.889
Error	0.020		

kD), representing glycoisoforms of the protein (Monsma and Wolfner 1988; Monsma *et al.* 1990; Park and Wolfner 1995). When Acp26Aa is transferred to females all the glycoisoforms undergo parallel processing involving proteolytic cleavage at or near conserved sites for a kexin-like protease (Park and Wolfner 1995). With this as reference, expression of Acp26Aa in DSR and DSRT males, and in females after mating within and between these two strains, was examined by immunoblotting using affinity-purified antibodies specific for Acp26Aa (Monsma and Wolfner 1988). The antibodies recognized a single band of apparent molecular weight of 34.5 in extracts of DSR and DSRT males (Figure 6, lanes 1 and 2). The band was of comparable intensity in males of the two strains, and multiple glycoisoforms were not observed. Reproductive tracts of unmated DSR or DSRT females did not contain detectable Acp26Aa (Figure 5, lanes 3 and 4). Acp26Aa was transferred to females during mating, and ~30 min later a processed band of 24.5 kD was detected in the mated females (Figure 6, lanes 5 and 6). This band was present with comparable intensity in females from both reciprocal crosses. We also observed that Acp36DE was present at comparable levels in males of both strains and transferred in equivalent amounts in the reciprocal matings (data not shown). Thus, the presence or absence of Wolbachia in males or females does not affect the production, transfer, or processing of the Acps we examined.

DISCUSSION

Previous studies of Wolbachia / host biology have usually involved measurements of reduced egg hatch rates caused by early embryonic lethality, *i.e.*, CI and the consequences of various environmental and bacterial factors that influence Wolbachia distribution and CI expression (Hoffmann *et al.* 1986, 1990, 1994; O'Neill and Karr 1990; Turelli and Hoffmann 1991, 1995; Turelli *et al.* 1992; Boyle *et al.* 1993; Giordano *et al.* 1995; Bourtzis *et al.* 1996; Karr *et al.* 1998; Poinsot *et al.* 1998; Feder *et al.* 1999).

Previous work, however, has not focused on direct fitness consequences of infection on the male, *per se.* We analyzed the relative fertility of DSR females mated with either DSRT or DSR males and have demonstrated

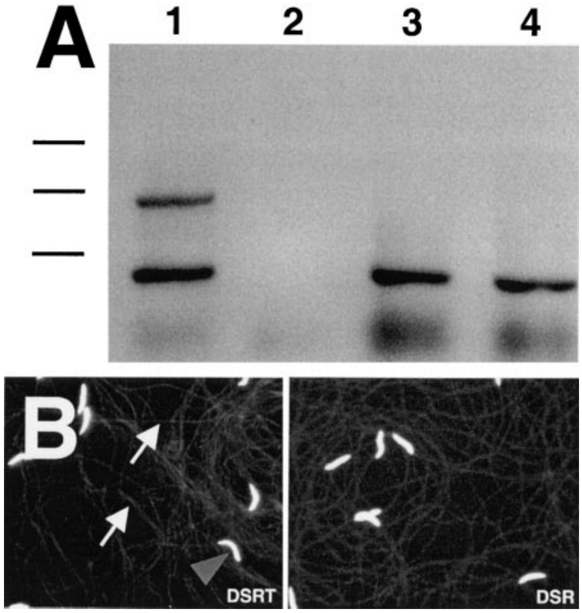

Figure 5.—Wolbachia are absent from mature sperm. (A) PCR analysis of testes and mature sperm. Testes were dissected from 3- to 5-day-old virgin DSR males and DNA amplified using 16S and 12S rDNA specific primers for Wolbachia and mitochondrial DNA sequences, respectively (lane 1), or from sperm isolated from DSR (lane 3) or DSRT (lane 4). (B) Sperm, dissected from 3- to 5-day-old DSR (left) and DSRT (right) males were fixed and stained with DAPI and viewed under epifluorescence. A brightly stained sperm head (arrow) connected to the weakly staining sperm tail (arrowheads) is shown in A and B.

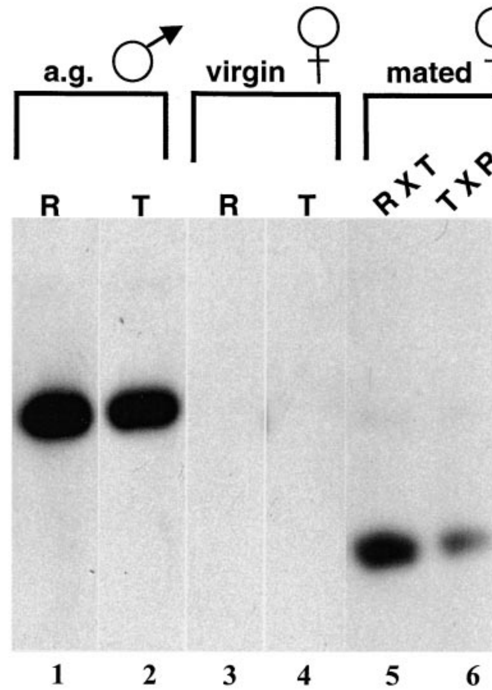

Figure 6.—Acp26Aa is made, transferred, and processed equally in DSR, DSRT, and their matings. Lanes 1 and 2 contain extracts of 10 accessory glands from DSR (R) and DSRT (T) *D. simulans* males, respectively. Note that the amount and apparent molecular weight (34.5 kD) of Acp26Aa in the two strains is equivalent. The signal from the *D. simulans* flies is ~10-fold less intense than that from the same number of *D. melanogaster* males (run on the same gel; not shown). We do not know whether this reflects interspecific differences in amounts of Acp26Aa or reflects the high sequence variation in this gene (Aguade 1998; Tsaur *et al.* 1998) resulting in low cross-reactivity of the *simulans* protein with the anti-*melanogaster* antibodies. Lanes 3 and 4 contain extracts of 10 unmated females from the same two strains, respectively. Lanes 5 and 6 contain extracts of 10 females taken ~30 min after mating from (lane 5) a compatible cross (DSR female × DSRT male) and (lane 6) an incompatible cross (DSRT female × DSR male). In both cases, a processed version of Acp26Aa (apparent molecular weight 24.5 kD) is seen.

a fitness effect on infected males. This fitness component is in addition to, and independent of, the expression of CI. Infected males paired with either a single compatible female or after sequential mating to multiple compatible females had significantly lower fertility than DSRT males.

The fertility difference is not related to any effect of Wolbachia on Acps 26Aa and 36DE. We found that the production and transfer of these Acps in DSR and DSRT males and the processing of these Acps in females after mating were similar. Sequential male mating by some Drosophila species results in decreased fertility (Lefevre and Jonsson 1962; Pitnick and Markow 1994) that can be reestablished after a 24-hr "recovery" period (Markow *et al.* 1978; Karr *et al.* 1998). Previous work suggested that the decreased fertility is due to the depletion of accessory gland secretions, resulting in the transfer of no (Lefevre and Jonsson 1962) to few (Hihara 1981) sperm. Supporting this interpretation, decreased fertility is seen in females mated to transgenic males that produce only 1% of the total amount of Acps compared to control males (Kalb *et al.* 1993). Although these males transfer close to normal amounts of sperm (Tram and Wolfner 2000), their sperm are not stored properly because of a lack of Acp36De (Neubaum and Wolfner 1999) and potentially other Acps. Reduced fertility in sequentially mated DSR males therefore could be possible because Acps are not transferred and/or processed correctly in the female. We doubt this is likely for several reasons.

First, virgin infected and uninfected males produce the same amounts of Acp26Aa (Figure 6) and Acp36DE (data not shown) and transfer the same amounts in a single mating. For multiply mated infected males to experience a fitness cost due to Acp depletion, the relative amounts of Acps transferred by the male and/or processed by the female would have to differ from similarly mated uninfected males. The fact that virgin males produce similar quantities of Acps argues against these possibilities. Additionally, Acp26Aa induces ovulation in newly mated females (Herndon and Wolfner 1995; Heifetz *et al.* 2000), so if females receive and process different amounts of this Acp on the basis of the infection status of the male, then females mated to DSR males should oviposit fewer eggs or take longer to oviposit than females mated to DSRT males. We found that DSR females mated to either DSR or DSRT males oviposited the same number of eggs over the same time (Figure 4a), but only females mated to DSR males produced a significantly larger proportion of unhatched eggs (Figure 4b). These results support our conclusions that Acps are not related to the fertility difference between infected and uninfected males and that Wolbachia infection does not influence Acp quantity or quality under the conditions tested.

Infected males produced fewer sperm cysts than uninfected males. The absence of Wolbachia from mature sperm and the lack of evidence indicating Wolbachia affects Acp production and processing suggests that the physiological cost to males is a direct consequence of its effect on sperm production. The discrepancy between infected and uninfected males in V-cyst production was greatest when males were 3 and 5 days old. Infected males did not increase the number of sperm being produced until they were 7 days old. Given that each sperm cyst contains 64 sperm, the effect of Wolbachia on sperm production could result in DSR males producing ~3000 fewer sperm than DSRT over the course of the experiment. We suggest that the fertility reduction of infected males seen in both single-pair and multiple mating conditions is due to Wolbachia's effect on sperm production, perhaps because infected males transferred fewer sperm. Males transfer ~3000 sperm in a single mating (Kaufman and Demerec 1942; Gilbert 1981). On no single day did DSR males produce 3000 fewer sperm than DSRT males. However, our sperm cyst protocol did not take into account mature sperm that were already past the V-cyst stage and in the seminal vesicles. Because sperm production in infected males is both decreased and slowed (see below), DSR males likely had far fewer sperm than DSRT. Future studies will focus on determining the number of sperm that infected and uninfected males transfer and females store to directly test if DSR males experience a fitness cost due to decreased sperm numbers.

The proximate mechanism by which Wolbachia depresses sperm production is unknown. A simple explanation for the observed differences may be the energy and time required by the cyst to remove Wolbachia during spermatid elongation and individualization. Wolbachia localize to the distal tip of the sperm cyst as sperm axonemes elongate, forming a "wave" of Wolbachia aligned immediately in front of the advancing sperm plasma membranes (Figure 1D). Because sperm axonemes grow to >1 mm in length in *D. simulans*, Wolbachia may provide a significant impediment to the rate of sperm growth during this period. Several lines of evidence support this interpretation. First, we found that infected males did not produce equivalent numbers of sperm cysts compared to DSRT until males were 7 days old, suggesting some impediment to growth. Second, 10-day-old males decreased V-cyst production significantly compared to days 5 and 7. The nature of this reduction is unidentified, but is presumably related to either a resorption of older sperm cysts or resorption and a concomitant reduction in the rate of stem cell divisions at the apical end of the testes. While both DSR and DSRT males experience this decrease, the reduction is more extreme in DSR, indicating DSR males experience greater resorption and/or slower rate of stem cell divisions. These results further support the idea that Wolbachia impede the rate of spermatogenesis.

In addition to the effect on male fertility, male multiple mating also abates the expression of CI (Karr *et al.* 1998). These studies suggested that Wolbachia-infected males that are multiply mated have differential sperm "quality" (with respect to the expression of CI) and that this difference is caused by reduction in the total residence time of sperm in testes. Since spermatogenesis begins in the gonadal imaginal disk in first-instar larvae and continues throughout the remaining larval, pupal, and adult stages (Fuller 1993), spermatocytes that begin development during these different stages of the life cycle are exposed to Wolbachia for different amounts of time during different developmental stages. One consequence of these differences is an increased residence time in the presence of Wolbachia for sperm that begin development during the first-instar stage. Presumably, the increased residence time would provide additional opportunity for Wolbachia to alter sperm function. Multiple male mating will cause males to transfer sperm more frequently, leading to the prediction that sperm would have decreased residence time in the testis, thereby decreasing CI expression. Thus, results presented in this article and in previous work on the role multiple male mating plays in CI expression (Karr *et al.* 1998) suggest that Wolbachia infection influences male fertility in two opposing ways: multiply mated infected males will have higher fitness due to decreased CI expression, but will have lower fitness, relative to an uninfected male, due to decreased sperm production.

Wolbachia could also act on sperm production by affecting Hsp expression. Hsp90 provides an essential function during spermatogenesis (Yue *et al.* 1999). Mutant *D. melanogaster* males producing abnormally low levels of Hsp90 have sperm bearing several ultrastructural and functional defects in microtubule-mediated processes that render these males sterile (Yue *et al.* 1999). Several aspects of the cell biology of CI suggest an association with microtubule-based processes or Hsps. First, in Wolbachia-induced incompatibility, paternal chromosomes do not fuse with maternal chromosomes in the egg and are associated with alterations in the mitotic spindle (Callaini *et al.* 1996, 1997; Lassy and Karr 1996). Second, infected males may exhibit low or absent Hsp expression (Feder *et al.* 1999), and infected flies raised continously under increased, but sublethal, temperatures result in loss of Wolbachia and CI expression (Hoffmann *et al.* 1986, 1990). Finally, brief heat shock of laboratory third-instar larvae results in decreased CI expression (Feder *et al.* 1999). These data suggest Wolbachia elicits CI due to modifications in sperm function through alterations in Hsps, microtubule-related functions, or interactions between these components.

Here we found that brief heat-shock treatment of male larvae elevates V-cyst production in infected males (hsDSR) typically to that of uninfected, non-heat-shocked males (DSRT). The effect of heat shock is greater on infected males than on uninfected males. Relative to their non-heat-shocked counterparts, infected males increased sperm production by 17% compared to 5% for uninfected males. On the basis of differences in sperm cyst numbers, over the course of the experiment, hsDSR males produced 1400 more sperm than DSR males (mean number of V cysts: hsDSR, 152.8; DSR, 130.7), whereas hsDSRT males produced only 615 more sperm than DSRT males (mean number of V cysts: hsDSRT, 188.5; DSRT, 178.9). Thus, heat shock may elevate the number of sperm such that infected males overcome the potential fitness cost of infection. The molecular mechanisms of heat shock on sperm production is unknown.

Our results in combination with others (Karr *et al.* 1998; Feder *et al.* 1999; Yue *et al.* 1999) suggest that CI abatement under conditions of heat shock occurs as a result of increased sperm production, thus reducing sperm residence time in Wolbachia-infected testes and subsequently decreasing the effect of infection on sperm function and male fertility. However, when males are exposed to room temperature, sperm production decreases, subsequently increasing the residency time of developing sperm in the presence of Wolbachia and thus increasing CI. The three related elements of heat shock, the subsequent increased sperm production, and concomitant decreased sperm residence time combine to decrease CI expression and recover fertility of infected males. Future work will examine whether heat shock ameliorates the cost of infection to males and restores fertility by elevating sperm production and/or affecting Acp production and processing.

Theoretical models of the spread of infection typically overestimate the realized dynamics of infection in natural populations of Drosophila (Hoffmann *et al.* 1990; Turelli and Hoffmann 1991, 1995). Several hypotheses have been suggested to explain this discrepancy, including the occurrence of natural antibiotics (Wade and Stevens 1985), imperfect maternal transmission (Hoffmann *et al.* 1990, 1998; Turelli and Hoffmann 1995), high temperature (Hoffmann *et al.* 1990) and heat shock (Feder *et al.* 1999), deleterious fecundity effects of infection (Hoffmann *et al.* 1990; Nigro 1991), male age (Hoffmann *et al.* 1990), larval density (Sinkins *et al.* 1995), and increased mating frequency by males (Karr *et al.* 1998). Mechanisms by which some of the above factors suppress CI expression have been suggested. For example, decreases in CI expression observed with increasing male age is correlated with loss of bacteria in spermatocysts (Bressac and Rousset 1993). As discussed above, increased mating frequency by males may abate CI by decreasing the residence time of sperm in the presence of CI-inducing Wolbachia (Karr *et al.* 1998). We propose that behavioral and ecophysiological conditions, such as multiple male mating and heat shock, respectively, may also contribute to the discrepancy between theoretical models and natural population dynamics of infection in two ways not previously appreciated. First, multiple mating by infected males, in the absence of heat shock, will deplete limited sperm resources because they produce fewer sperm than uninfected males. This decreased sperm production subsequently lowers fitness and may decrease the number of infected individuals since infected females (that vertically transmit the infection) mated to infected males would also experience decreased fitness (Figures 3 and 4b). Second, while heat shock increases sperm production rates of infected males, sperm production rates of uninfected males also increase, thereby further increasing the potential fitness of uninfected males relative to infected males.

These results suggest a series of intriguing experiments designed to further elucidate the role Wolbachia plays in manipulating reproduction and reproductive success in its hosts and the subsequent population dynamics of infection. For example, within a species and across species, there is variable expression in the severity of CI and its effect on egg production (O'Neill and Karr 1990; Hoffmann *et al.* 1994; Giordano *et al.* 1995; Bourtzis *et al.* 1996). Do species that have low or nonexistent CI expression, but carry the bacteria, experience decreased sperm production that influences subsequent reproductive success? Hoffmann *et al.* (1994) found that infected sperm of a *D. melanogaster* strain with low CI expression were at a disadvantage in sperm competition compared to uninfected sperm. They suggested that infected sperm may be lost more readily from females when an infected male is the first mate of a female competing against an uninfected second male. Our present results suggest, alternatively, that there may not be as many sperm in storage from that infected first male because he produces fewer sperm and, thus, females mated to those males store fewer sperm. If true, infected individuals will be at a disadvantage and the infection should not go to fixation in the population.

In addition, transinfection experiments of a Wolbachia-infected, but non-CI-expressing, population of *D. mauritiana* into an uninfected *D. simulans* strain failed to ellicit CI expression (Giordano *et al.* 1995). The ability of Wolbachia to infect *D. mauritiana* with no corresponding CI expression and the lack of inducible CI expression in *D. simulans* begs the question of what effect the bacteria have on sperm production and subsequent reproductive success in both species. Similarly, the low level of CI expression in *D. melanogaster* and the failure of a selection experiment designed to increase CI has been suggested to reflect selection on the host to overcome the costs of the bacteria (Hoffmann *et al.* 1994). Perhaps sperm production in these cases is not influenced. Alternatively, given that there is high CI expression in uninfected DSR transinfected with Wolbachia from *D. melanogaster,* these transinfected individuals should have decreased sperm production associated with the challenge of infection. In support of this prediction, bacterial load in the spermatocytes of transinfected DSR was 10 times higher than that found in *D. melanogaster* (Poinsot *et al.* 1998).

Finally, in all the above scenarios, what is the result of heat shock on sperm production and any corresponding changes in reproductive success? Clearly, determining interactions between infection status, the severity of CI expression, heat shock, and fitness will further elucidate the distribution of Wolbachia and host effects.

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LITERATURE CITED

Aguade, M., 1998 Different forces drive the evolution of the *Acp26Aa* and *Acp26Ab* accessory gland genes in the *Drosophila melanogaster* species complex. Genetics **150:** 1079–1089.

Aigaki, T., I. Fleischmann, P.-S. Chen and E. Kubli, 1991 Ectopic expression of sex peptide alters reproductive behavior of female *Drosophila melanogaster.* Neuron **7:** 557–563.

Bertram, M. J., D. M. Neubaum and M. F. Wolfner, 1996 Localization of the Drosophila male accessory gland protein Acp36DE in the mated female suggests a role in sperm storage. Insect Biochem. Mol. Biol. **26:** 971–980.

Bouchon, D., T. Rigaud and P. Juchault, 1998 Evidence for widespread Wolbachia infection in isopod crustaceans: molecular identification and host feminization. Proc. R. Soc. Lond. Ser B: Biol. Sci. **265:** 1081–1090.

Bourtzis, K., A. Nirgianaki, G. Markakis and C. Savakis, 1996 Wolbachia infection and cytoplasmic incompatibility in Drosophila species. Genetics **144:** 1063–1073.

Boyle, L., S. L. O'Neil, H. M. Robertson and T. L. Karr, 1993 Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila.* Science **260:** 1796–1799.

Breeuwer, J. A., and J. H. Werren, 1990 Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. Nature **346:** 558–560.

Breeuwer, J. A., and J. H. Werren, 1993 Cytoplasmic incompatibility and bacterial density in *Nasonia vitrapennis.* Genetics **135:** 565–574.

Bressac, C., and F. Rousset, 1993 The reproductive incompatibility system in *Drosophila simulans*: DAPI-staining analysis of the *Wolbachia* symbionts in sperm cysts. J. Invert. Pathol. **61:** 226–230.

Callaini, G., M. G. Riparbelli, R. Giordano and R. Dallai, 1996 Mitotic defects associated with cytoplasmic incompatibility in Drosophila simulans. J. Invert. Pathol. **67:** 55–64.

Callaini, G., R. Dallai and M. G. Riparbelli, 1997 Wolbachia-induced delay of paternal chromatin condensation does not prevent maternal chromosomes from entering anaphase in incompatible crosses of Drosophila simulans. J. Cell Sci. **110:** 271–280.

Chen, P. S., E. Stumm-Zollinger, T. Aigaki, J. Balmer, M. Bienz *et al.*, 1988 A male accessory gland peptide that regulates reproductive behavior of female *D. melanogaster.* Cell **54:** 291–298.

Clark, A. G., M. Aguade, T. Prout, L. G. Harshman and C. H. Langley, 1995 Variation in sperm displacement and its association with accessory gland protein loci in *Drosophila melanogaster.* Genetics **139:** 189–201.

Feder, M. E., T. L. Karr, W. Yang, J. M. Hoekstra and A. C. James, 1999 Interaction of *Drosophila* and its endosymbiont *Wolbachia*: natural heat shock and the overcoming of sexual incompatibility. Am. Zool. **39:** 363–373.

Fuller, M. T., 1993 Spermatogenesis, pp. 71–147 in *The Development of Drosophila melanogaster,* edited by M. Bate and A. M. Arias. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Gilbert, D. G., 1981 Ejaculate esterase 6 and initial sperm use by female *Drosophila melanogaster.* J. Insect Physiol. **27:** 641–650.

Giloh, H., and J. W. Sedat, 1982 Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by *n*-propyl gallate. Science **217:** 1252–1255.

Giordano, R., S. L. O'Neill and H. M. Robertson, 1995 Wolbachia infections and the expression of cytoplasmic incompatibility in *Drosophila sechellia* and *D. mauritiana.* Genetics **140:** 1307–1317.

Glaser, R. L., M. F. Wolfner and J. T. Lis, 1986 Spatial and temporal pattern of hsp26 expression during normal development. EMBO J. **5:** 747–754.

Glover, D. M., J. Raff, T. L. Karr, S. L. O'Neill, H. Lin *et al.*, 1990 Parasites in Drosophila embryos [letter; comment]. Nature **348:** 117.

Gromko, M. H., and T. A. Markow, 1993 Courtship and remating in field populations of *Drosophila.* Anim. Behav. **45:** 253–262.

Gromko, M. H., M. E. A. Newport and M. G. Kortier, 1984 Sperm dependence of female receptivity to remating in *Drosophila melanogaster.* Evolution **38:** 1273–1282.

Habrova, V., M. Takac, J. Navratil, J. Macha, N. Ceskova *et al.*, 1996 Association of rous sarcoma virus DNA with Xenopus laevis spermatozoa and its transfer to ova through fertilization. Mol. Reprod. Dev. **44:** 332–342.

Heifetz, Y., O. Lung, E. A. Frongillo and M. F. Wolfner, 2000 The Drosophila seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary. Curr. Biol. **10:** 99–102.

Herndon, L. A., and M. F. Wolfner, 1995 A Drosophila seminal fluid protein, Acp26Aa, stimulates egg laying in females for 1 day after mating. Proc. Natl. Acad. Sci. USA **92:** 10114–10118.

Hihara, F., 1981 Effects of the male accessory gland secretion on oviposition and remating in females of *Drosophila melanogaster.* Zool. Mag. **90:** 307–316.

Hihara, F., and H. Kurokawa, 1987 The sperm length and the internal reproductive organs of *Drosophila* with special references to phylogenetic relationships. Zool. Sci. **4:** 167–174.

Hoffmann, A. A., 1985 Male effects on fecundity in *Drosophila melanogaster.* Evolution **39:** 638–644.

Hoffmann, A. A., M. Turelli and G. M. Simmons, 1986 Unidirectional incompatibility between populations of *Drosophila simulans.* Evolution **40:** 692–701.

Hoffmann, A. A., M. Turelli and L. G. Harshman, 1990 Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans.* Genetics **126:** 933–948.

Hoffmann, A. A., D. J. Clancy and E. Merton, 1994 Cytoplasmic incompatibility in Australian populations of Drosophila melanogaster. Genetics **136:** 993–999.

Hoffmann, A. A., M. Hercus and H. Dagher, 1998 Population dynamics of the Wolbachia infection causing cytoplasmic incompatibility in Drosophila melanogaster. Genetics **148:** 221–231.

Kalb, J. M., A. J. DiBenedetto and M. F. Wolfner, 1993 Probing the function of Drosophila melanogaster accessory glands by directed cell ablation. Proc. Natl. Acad. Sci. USA **90:** 8093–8097.

Karr, T. L., 1996 Paternal investment and intracellular sperm-egg interactions during and following fertilization in Drosophila. Curr. Topics Dev. Biol. **34:** 89–115.

Karr, T. L., W. Yang and M. E. Feder, 1998 Overcoming cytoplasmic incompatibility in Drosophila. Proc. R. Soc. Lond. Ser B: Biol. Sci. **265:** 391–395.

Kaufman, B. P., and M. Demerec, 1942 Utilization of sperm by the female *Drosophila melanogaster*. Am. Nat. **76:** 445–469.

Kose, H., and T. L. Karr, 1995 Organization of *Wolbachia pipientis* in the *Drosophila* fertilized egg and embryo revealed by an anti-Wolbachia monoclonal antibody. Mech. Dev. **51:** 275–288.

Lassy, C. W., and T. L. Karr, 1996 Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila simulans*. Mech. Dev. **57:** 47–58.

Lefevre, G., Jr., and U. B. Jonsson, 1962 Sperm transfer, storage displacement and utilization in *Drosophila melanogaster*. Genetics **47:** 1719–1736.

Markow, T. A., M. Quaid and S. Kerr, 1978 Male mating experience and competitive courtship success in *D. melanogaster*. Nature **276:** 821–822.

Monsma, S. A., and M. F. Wolfner, 1988 Structure and expression of a Drosophila male accessory gland gene whose product resembles a peptide pheromone precursor. Genes Dev. **2:** 1063–1073.

Monsma, S. A., H. A. Harada and M. F. Wolfner, 1990 Synthesis of two Drosophila male accessory gland proteins and their fate after transfer to the female during mating. Dev. Biol. **142:** 465–475.

Neubaum, D. M., and M. F. Wolfner, 1999 Mated *Drosophila melanogaster* females require a seminal fluid protein, Acp36DE, to store sperm efficiently. Genetics **153:** 845–857.

Nigro, L., 1991 The effect of heteroplasmy on cytoplasmic incompatibility in transplasmic lines of Drosophila simulans showing a complete replacement of the mitochondrial DNA. Heredity **66:** 41–45.

O'Neill, S. L., and T. L. Karr, 1990 Bidirectional incompatabilty between conspecific populations of *Drosophila simulans*. Nature **348:** 178–180.

O'Neill, S. L., R. Giordano, A. M. Colbert, T. L. Karr and H. M. Robertson, 1992 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. USA **89:** 2699–2702.

Park, M., and M. F. Wolfner, 1995 Male and female cooperate in the prohormone-like processing of a Drosophila melanogaster seminal fluid protein. Dev. Biol. **171:** 694–702.

Pitnick, S., and T. A. Markow, 1994 Male gametic strategies: sperm size, testes size, and the allocation of ejaculate among successive mates by the sperm-limited fly *Drosophila pachea* and its relatives. Am. Nat. **143:** 785–819.

Poinsot, D., K. Bourtzis, G. Markakis, C. Savakis and H. Mercot, 1998 Wolbachia transfer from *Drosophila melanogaster* into *D. simulans*: host effect and cytoplasmic incompatibility relationships. Genetics **150:** 227–237.

Reed, K. M., and J. H. Werren, 1995 Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (Wolbachia): a comparative study of early embryonic events. Mol. Reprod. Dev. **40:** 408–418.

Rousset, F., D. Bouchon, B. Pintureau, P. Juchault and M. Solignac, 1992 Wolbachia endosymbionts responsible for various alterations of sexuality in arthropods. Proc. R. Soc. Lond. Ser B: Biol. Sci. **250:** 91–98.

Schwartz, J. M., and C. R. B. Boake, 1992 Sexual dimorphism in remating in Hawaiian *Drosophila* species. Anim. Behav. **44:** 231–238.

Simmons, L. W., and M. Siva-Jothy, 1998 Sperm competition in insects: mechanisms and the potential for selection, pp. 341–434 in *Sperm Competition and Sexual Selection*, edited by T. R. Birkhead and A. P. Møller. Academic Press, London.

Sinkins, S. P., H. R. Braig and S. L. O'Neill, 1995 Wolbachia superinfections and the expression of cytoplasmic incompatibility. Proc. R. Soc. Lond. Ser B: Biol. Sci. **261:** 325–330.

Stouthamer, R., J. A. Breeuwert, R. F. Luck and J. H. Werren, 1993 Molecular identification of microorganisms associated with parthenogenesis. Nature **361:** 66–68.

Tram, U., and M. F. Wolfner, 1999 Male seminal fluid proteins are essential for sperm storage in *Drosophila melanogaster*. Genetics **153:** 837–844.

Tsaur, S. C., C. T. Ting and C. I. Wu, 1998 Positive selection driving the evolution of a gene of male reproduction, Acp26Aa, of Drosophila. II. Divergence versus polymorphism. Mol. Biol. Evol. **15:** 1040–1046.

Turelli, M., and A. A. Hoffmann, 1991 Rapid spread of an inherited incompatible factor in *Drosophila*. Nature **353:** 440–442.

Turelli, M., and A. A. Hoffmann, 1995 Cytoplasmic incompatibility in Drosophila simulans: dynamics and parameter estimates from natural populations. Genetics **140:** 1319–1338.

Turelli, M., and H. A. Orr, 1995 The dominance theory of Haldane's rule. Genetics **140:** 389–402.

Turelli, M., A. A. Hoffmann and S. W. McKechnie, 1992 Dynamics of cytoplasmic incompatibility and mtDNA variation in natural *Drosophila simulans* populations. Genetics **132:** 713–723.

Wade, M. J., and L. Stevens, 1985 Microorganism mediated reproductive isolation in flour beetles (genus Tribolium). Science **227:** 527–528.

Werren, J. H., D. Windsor and L. Guo, 1995a Distribution of Wolbachia among neotropical arthropods. Proc. R. Soc. Lond. Ser B: Biol. Sci. **262:** 197–204.

Werren, J. H., W. Zhang and L. R. Guo, 1995b Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods. Proc. R. Soc. Lond. Ser B: Biol. Sci. **261:** 55–63.

Yen, J. H., and A. R. Barr, 1973 The etiological agent of cytoplasmic incompatibility in Culex pipiens. J. Invert. Pathol. **22:** 242–250.

Yue, L., T. L. Karr, D. F. Nathan, H. Swift, S. Srinivasan *et al.*, 1999 Genetic analysis of viable Hsp90 alleles reveals a critical role in Drosophila spermatogenesis. Genetics **151:** 1065–1079.

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